

REVIEW



Pathophysiological functions of Rnd proteins

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ABSTRACT

Rnd proteins constitute a subfamily of Rho GTPases represented in mammals by Rnd1, Rnd2 and Rnd3. Despite their GTPase structure, their specific feature is the inability to hydrolyse GTP-bound nucleotide. This aspect makes them atypical among Rho GTPases. Rnds are regulated for their expression at the transcriptional or post-transcriptional levels and they are activated through post-translational modifications and interactions with other proteins. Rnd proteins are mainly involved in the regulation of the actin cytoskeleton and cell proliferation. Whereas Rnd3 is ubiquitously expressed, Rnd1 and 2 are tissue-specific. Increasing data has described their important role during development and diseases. Herein, we describe their involvement in physiological and pathological conditions with a focus on the neuronal and vascular systems, and summarize their implications in tumorigenesis.

ARTICLE HISTORY

Received 1 June 2020
Revised 2 September 2020
Accepted 24 September 2020

KEYWORDS

Rnd; pathophysiology;
neuronal system; vascular
system; cancer

1. Rnd, an atypical Rho GTPase family

1.1 The Rho GTPase family in human and emergence of Rnd during evolution

The Rho family of GTPases belongs to the Ras superfamily of small GTPases and contains, in humans, 20 members divided into 8 subfamilies according to their sequence homology: RhoA, Rac, Cdc42, RhoU/V, RhoD/F, RhoH, RhoBTB and Rnd. One important and common feature of Rho GTPase proteins is their ability to bind nucleotides and to cycle between GDP-bound (OFF) inactive state and GTP-bound (ON) active state. The switch between the two states occurs when the gamma phosphate group of the GTP is hydrolysed. The change in the structure of the GTPase on hydrolysis reduces binding to downstream effectors and turns off the protein. For most of Rho family members, three types of proteins regulate the transition between their ON and OFF states. Guanine nucleotide exchange factors (GEFs) play as activators allowing the replacement of GDP by GTP whereas GTPase-activating proteins (GAPs) inactivate them by promoting the GTP hydrolysis to GDP. Additional regulation is provided by Guanine nucleotide dissociation inhibitors (GDIs) which sequester GTPases away from their active subcellular compartments and prevent both nucleotide exchange and GTP hydrolysis. Within the RhoGTPase family, the Rnd subfamily is defined by three members sharing sequence identity. The Rnd

subfamily consists of three proteins: Rnd1/Rho6/RhoS, Rnd2/Rho7/RhoN and Rnd3/Rho8/RhoE (Table 1). The human *RND1* gene is located on chromosome 12 (q12-q13), while the *RND2* and *RND3* genes are located, respectively, on chromosome 17 (q21) and on chromosome 2 (q23). Rnd protein family emerged in the ancestral chordates, only expressed in vertebrates and shows a relative late evolution of history. This finding suggests that Rnd proteins are implicated in vertebrate-specific biological mechanisms [1].

1.2 Specific features of Rnd proteins

Foster and collaborators identified the first member of the Rnd family, called RhoE by yeast two-hybrid screen with the GAP, p190RhoGAP-A (encoded by the *ARHGAP35* gene), as bait [2] (Table 2). They showed by sequence alignments that RhoE protein has similarity to different members of the Rho GTPase family and exhibits strong homology to the mammalian RhoA, RhoB and RhoC (54% identity). Nevertheless, RhoE was described as an unusual member of Rho family existing only in the GTP-bound form and lacking intrinsic GTPase activity. It is resistant to RhoGAP activity; incubation of GTP-loaded RhoE with purified preparation of p190RhoGAP or Cdc42GAP failed to promote any detectable GTP hydrolysis by RhoE [2]. Two years later, the complete human cDNA for three members of the same family was obtained. These new members were nominated Rnd

Table 1. Specific features of Rnd proteins.

	Rnd1	Rnd2	Rnd3	Classical Rho members (i.e. Rho/Rac/Cdc42)
Other names	ARHS, Rho6, RhoS	ARHN, Rho7, RhoN	ARHE, Rho8, RhoE, MemB	
Locus (Chromosome/cytoband)	12/q13:12	17/q21:31	2/q23:3	
Total length (aa)	232	227	244	
N-ter	+ 11 aa*	+ 5aa*	+ 21 aa*	
Cter insert	+ 30 aa	+ 31 aa	+ 32 aa	
MW	26 kDa	26 kDa	27 kDa	± 21–22 kDa
G14/G12	V	A	S	G14 (RhoA), G12 (Rac1/Cdc42)
Q63/Q61 (switchII)	S	S	S	Q63 (RhoA), Q61 (Rac1/Cdc42)
GTPase activity	none	none	none	yes
CAAX box	CSIM	CNLM	CTVM	CLAV(V/L)L
Post-translational modification	Farnesyl group (15 carbons lipid motif) Phosphorylation Tissue specific	Farnesyl group (15 carbons lipid motif) Phosphorylation Tissue specific	Farnesyl group (15 carbons lipid motif) Phosphorylation Ubiquitous	geranyl-geranyl group (20 carbons lipid motif) Phosphorylation Ubiquitous
Expression				

(‘round’) for their ability to induce cell rounding [3]. Like RhoE, renamed Rnd3, Rnd1 and Rnd2 have altered sequences at residues critical for GTP hydrolysis (mainly serine instead of the conserved glutamine in classical GTPases) and are permanently in the GTP-bound form with no detectable GTPase activity (Table 1). As previously described for pseudokinases that lack kinase activity, Rnd proteins are considered pseudoGTPases. They indeed belong to a recently described pseudoGTPase subgroup (class ii) able to bind nucleotide but with no catalytic activity [4]. Rnd proteins share 54–63% identity and display substantial differences compared to the classical RhoGTPase molecular switches. The molecular weights of Rnd proteins (~26–27 kD) are higher than Rho/Rac/Cdc42 (~22kD) due to the NH2-terminal extensions (5–21 residues) and COOH-terminal extensions (about 30 residues) for all three (Table 1). Rnd proteins are positively charged with basic isoelectric point (pIs 8.1 to 8.7) whereas most Rho-family proteins have slightly acidic pIs. Unlike most other Rho proteins which have a C-terminus ‘CAAX box’ with leucine and are predominantly geranyl-geranylated, Rnd proteins are farnesylated at the last methionine residue [3,5]. The recognition of the CAAX mediated by farnesyltransferase (FT) and/or geranylgeranyltransferase type I (GGTase-I) is essential for all Rho family GTPases to undergo a series of posttranslational modifications promoting subcellular localization to the plasma membrane and/or endomembranes. Indeed, localization of all three Rnd is extremely sensitive to FT inhibitors (FTI) but not to GGTase-I inhibitors (GGTI) and resulted in the loss of plasma membrane localization, and increased cytoplasmic and nuclear accumulation of all three Rnd proteins. Thus, the subcellular localization of Rnd1 and Rnd3 proteins is predominantly plasma membrane-associated while Rnd2 lacks this association and appears to be in the cytosol and endosomes [6], directly bound to vacuolar protein sorting 1-A (Vps4-A), the central protein regulating early endosome trafficking [7] (Table 2). Interestingly, replacement of Rnd2 C-terminal domain with that of Rnd3 allowed Rnd2 to be localized at the plasma membrane and compensate for Rnd3 loss of function in cortical neurons [8]. The phosphorylation of Rnd3 by the serine threonine kinases ROCK1, protein kinase C (PKCα) or TAO kinase (TAOK1, TAOK2) induces the protein translocation from the plasma membrane to the cytosol [9–11]. Moreover, the interaction of the phosphorylated forms of Rnd1 and Rnd3 with 14-3-3 proteins also favours this translocation inhibiting Rnd-induced cell rounding [12]. Rnd1 is concentrated at point of cell-cell contacts in confluent 3T3 fibroblasts and in epithelial cells with a peripheral staining of Rnd1 colocalizing with pancytokeratin [3]. Similarly, Rnd3 was shown to localize to

Table 2. Rnd partners and associated functions.

Which Rnd?	Protein partner	Associated function(s)	References
Rnd1	Grb7	<i>unknown</i>	[17]
Rnd1	STI1	Neurite growth	[46]
Rnd1	Plexin B1	RhoA activation	[59]
Rnd1	Plexin A1	Cytoskeletal collapse	[61]
Rnd1	SCG10	Microtubule stability	[56]
Rnd1	FLTR1	Cadherin endosomal localization	[130]
Rnd1	Netrin receptor Unc5B	Cell adhesion, Xenopus development	[131]
Rnd2	Plexin D1	Inhibition of cortical neurons and R-Ras downregulation	[62]
Rnd2	Vps-4A	Endosomal trafficking	[7]
Rnd2	Pragmin	Neurite outgrowth inhibition	[50]
Rnd2	Rapostlin/FBP17	Neurite branching	[51]
Rnd2	Bacurd2/Tnfaip1	Neuronal migration	[32]
Rnd2	MgcRacGAP	Golgi-derived vesicle localization in male germ cells	[132]
Rnd3	ROCK1	Phosphorylation and cytosol localization	[11]
Rnd3	PKC	Phosphorylation and cytosol localization	[10]
Rnd3	TAOK1, TAOK2	Phosphorylation and cytosol localization	[9]
Rnd3	Plexin B2	Cortical neuron migration	[35,133]
		Cell rounding	
Rnd3	NF-kB (p50; p65)	NF-kB signalling inhibition	[89]
		Inflammatory response	
Rnd3	Skp2	Ubiquitinylation and degradation	[25]
Rnd1, Rnd2	FRS2 α / β	RhoA regulation via FGF receptor signalling	[48]
Rnd2, Rnd3	Bacurd1/Kctd13	Dendritic maturation of cerebral cortical neurons	[36]
	Bacurd2/Tnfaip1		
Rnd1, Rnd3	14-3-3	Cell rounding and cytosol localization	[12]
Rnd1, Rnd3	Socius	Loss stress fibres/suppresses cell rounding induced by Rnd	[134]
Rnd1, Rnd2, Rnd3	p190RhoGAPs (p190A/ARHGAP35; p190B/ARHGAP5)	RhoA inhibition	[2,135]
		Rnd protein stability	[24,25]
Rnd1, Rnd2, Rnd3	Syx/PLEKHG5	RhoA inhibition	[24,26]
		Rnd protein stability	

cell-cell contacts during tumour cell collective migration [13] and to cell-cell collision zones upon the migration of human cancer-associated fibroblasts in extracellular matrices [14]. Rnd3 protein was also found to localize with the Rho kinase ROCK1, one of its partner (Table 2), on the Golgi apparatus in COS-7 fibroblasts [15]. Finally, Rnd1 and Rnd3 were found associated to lipid rafts thanks to a KERRA (Lys-Glu-Arg-Arg-Ala) sequence present in their N-terminus in various cell lines including COS-7, HeLa, HEK-293 and NIH-3T3 cells [16].

1.3 Rnd expression and functions

Early studies have demonstrated that all three Rnds display distinct patterns of expression [2,3]. Recent RNA sequencing performed on adult human tissues confirmed those data (Figure 1). Rnd1 is expressed mostly in the liver and several brain tissues. Rnd2 is highly abundant in testis and in the brain. Rnd3 is ubiquitously expressed, showing expression in all tissues tested, with lower levels in the pancreatic tissue and bone marrow (Figure 1) [18]. In foetal brain, lung, kidney and liver tissues, the patterns of Rnds expression are similar to that observed in adults [3]. Thus, although the three Rnds show different pattern of expression, they are all expressed in the brain.

As described above, Rnd proteins are not regulated by the typical GDP/GTP cycle; thus, biogenesis and

decay of mRNA and proteins play an important role in their regulation. Besides intrinsic stability of each individual mRNA, transcriptional and post-transcriptional regulation of Rnds may change in response to a variety of extracellular stimuli. Indeed, it is now clear that all Rnd proteins are highly regulated and modifications of their expression in response to multiple different stimuli, such as growth factors, and conditions, such as hypoxia, are well documented [19–23]. At the protein level, no specific guanine nucleotide exchange factors or GTPase activating proteins for Rnds are known. Instead, Rnds are activated through post-translational modifications (i.e. phosphorylation) or interaction with other proteins and inactivated through cytosolic sequestration or ubiquitinylation and destruction by the proteasome [12,24,25]. For example, the interaction of Syx (also named PLEKHG5) via its ubiquitin-like ‘Rnd-binding domain’ (RndBD) with Rnd3 elevates the half-life of Rnd3, thus increasing Rnd3 protein stability (Table 2). The effect of Syx on Rnd3 is also observed on the other Rnd proteins, but the interaction of Syx with Rnd1 or Rnd2 is weaker than the one with Rnd3 [24]. Syx is also considered as an Rnd effector as Rnd proteins potentially modulate RhoA activation through this RhoGEF [26].

Over past years, members of the Rho GTPase family have been demonstrated as principal actors in many cellular processes including cytoskeletal

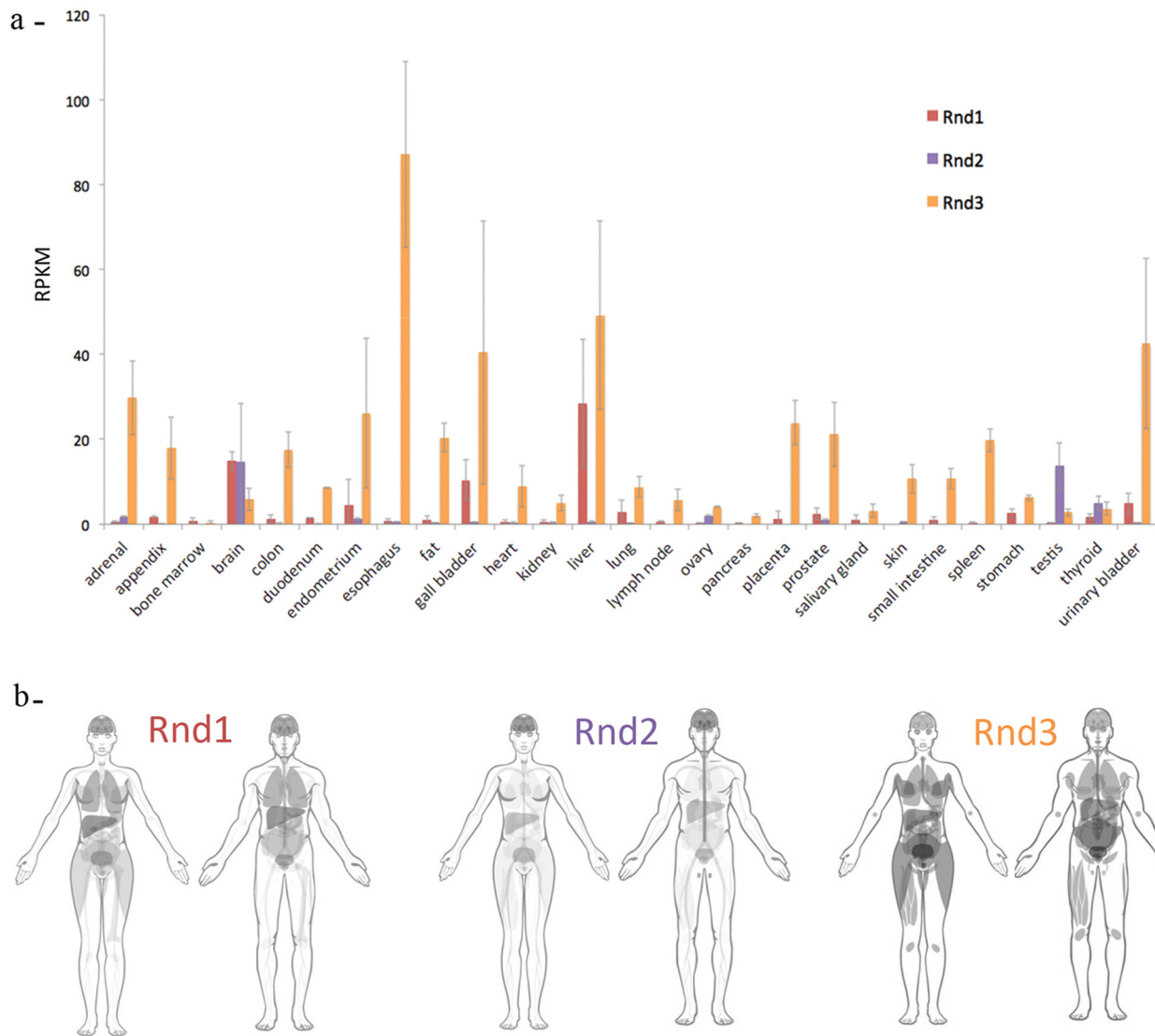


Figure 1. Expression of *RND* in healthy human tissues. A) Data extracted from the Gene database (<https://www.ncbi.nlm.nih.gov/gene>). RNA-seq was performed of tissue samples from 95 human individuals representing 27 different tissues in order to determine tissue-specificity of the three *RND* protein-coding genes (BioProject: PRJEB4337). The y axis shows RPKM (Reads Per Kilobase Million) reflecting expression level, whereas the x axis indicates human tissues. B) Anatomogram extracted from the human protein Atlas database, showing tissue mRNA expression for Rnd1 (<https://www.proteinatlas.org/ENSG00000172602-RND1/tissue>), Rnd2 (<https://www.proteinatlas.org/ENSG00000108830-RND2/tissue>), and Rnd3 (<https://www.proteinatlas.org/ENSG00000115963-RND3/tissue>). Image credit: Human Protein Atlas.

organization, cell cycle, proliferation, cell shape and movement. Rnd proteins have also been implicated in these processes, as they are known to act mainly as antagonists of the RhoA/ROCK pathway, even if they also function through specific effectors. Rnd regulation of these key cellular events was mainly described in the neuronal and vascular systems, and during tumorigenesis. Thus, in this review, we focus on the pathophysiological functions of Rnds in these three contexts.

2. Rnd proteins in the neuronal system

2.1 In physiological conditions

As mentioned previously, the three members of the Rnd family are highly expressed in the brain and over the past several years, it has become clear that these RhoGTPases play a central role in various aspects of neuronal development (Table 3). The development of a neuron is a multistep process that begins with its generation from a neuronal

Table 3. Summary of Rnd protein functions in neuronal development.

Rnd	Cell types	Functions	References
Rnd1	PC12 cells in culture	stimulation of neurite outgrowth through inhibition of RhoA signalling	[45,46,48]
	Hippocampal neurons in culture	promotion of axon formation	[56]
	Hippocampal neurons in culture	growth cone collapse through Rnd1-PlexinB1 interaction	[59] and [57]
Rnd2	Hippocampal neurons in culture	stimulation of dendritic growth and branching	[64]
	Hippocampal neurons in culture	promotion of spine formation and elongation	[66]
	Cortical neurons <i>in vivo</i>	promotion of migration, by regulating multipolar-to-bipolar transition in the intermediate zone, partially through RhoA inhibition	[32–34]
Rnd3	PC12 cells in culture	inhibition of neurite outgrowth through Pragma	[50]
	PC12 cells in culture	stimulation of neurite branching through Rapostlin	[51]
	Cortical neurons <i>in vivo</i>	inhibition of neurite outgrowth	[8,33]
	Cortical neurons in culture	inhibition of axon outgrowth through Rnd2-PlexinD1 interaction	[62]
	Hippocampal neurons in culture	promotion of spine formation	[53]
	NSCs in embryonic cerebral cortex <i>in vivo</i>	control of interkinetic nuclear migration; orientation of cleavage plane orientation and maintenance of adherens junction by inhibiting actin polymerization	[27]
	Basal progenitors in embryonic cerebral cortex <i>in vivo</i>	inhibition of proliferation through suppression of cyclinD1	[27]
	NSCs in postnatal SVZ <i>in vivo</i>	inhibition of proliferation through regulation of Notch signalling	[28,30]
	Cortical neurons <i>in vivo</i>	promotion of migration, by regulating locomotion and nucleus-centrosome coupling in the cortical plate, through RhoA inhibition	[8,35]
	Neuroblasts in rostral migratory stream <i>in vivo</i>	promotion of migration of neuroblasts from SVZ to olfactory bulb	[28]
	Retinal interneurons <i>in vivo</i>	promotion of correct positioning of ON/OFF starburst amacrine cells	[42]
	PC12 cells in culture	stimulation of neurite outgrowth through inhibition of RhoA signalling	[47]
	Hippocampal neurons in culture	stimulation of neurite outgrowth through inhibition of RhoA signalling	[49]
	Cortical neurons <i>in vivo</i>	inhibition of neurite outgrowth	[8]
	Hippocampal neurons in culture	promotion of axon outgrowth	[49]
	Hippocampal neurons in culture	promotion of spine formation through inhibition of RhoA signalling	[67]

progenitor cell residing in a germinal zone of the nascent brain. Then, after migration from its birth-place to its final location, the newborn neuron develops specialized processes, axon and dendrites, towards proper target regions and finally induces spine formation and synaptic connections with appropriate partners.

2.1.1 Rnd functions in neuronal progenitors

Cortical progenitors

So far, only Rnd3 has been described to play a role in neuronal stem cells/progenitors. In the embryonic cortex, using *in utero* electroporation of *Rnd3* shRNA, Rnd3 was shown, in radial glial cells (RGCs), to regulate the movement of the nuclei occurring during interkinetic nuclear migration (a process whereby nuclei change position along the apico-basal axis during the course of the cell cycle), to exert a control on the orientation of the cleavage plane and to be required for the maintenance of adherens junctions [27]. Importantly, Rnd3 regulates these early steps of neurogenesis by inhibiting actin filament polymerization since the defects observed after *Rnd3* knockdown are rescued when a constitutively active form of cofilin (cofilin^{S3A}), which disrupts actin filaments, is co-electroporated [27]. In addition, Rnd3 also limits the divisions of basal progenitors, but, interestingly, this

involves the suppression of cyclin D1 translation. Rnd3 thus exerts pleiotropic functions in cortical progenitors but employs distinct mechanisms in RGCs and basal progenitors.

SVZ progenitors

Besides these functions in cortical progenitors, data obtained with Rnd3 mutant suggest that Rnd3 also controls progenitor behaviour in the subventricular zone (SVZ) [28]. This zone along the lateral ventricle wall represents a reservoir of neural stem/progenitor cells in the postnatal and adult brain. There, neuroblasts are continuously produced by stem cells/progenitors and these neuroblasts then migrate through the rostral migratory stream (RMS) to reach the olfactory bulb (OB) where they differentiate in two principal cell types, granule cells and periglomerular cells [29]. Using BrdU labelling, Ballester-Lurbe and colleague observed an excessive proliferation in the SVZ at postnatal day 9 (P9) in the Rnd3 mutants compared to control mice, but not at birth [28]. This phenotype can be attributed to enhanced Notch signalling, which acts in coordination with proneural transcription factors to balance proliferation and differentiation of NSCs. Increased levels of the activated form of Notch (NICD: Notch intracellular domain) and of the Notch target *Hes1* have been reported in the postnatal SVZ of *Rnd3* knockouts and this increase is what drives the aberrant growth of

NSCs, since Notch inhibition abolished the phenotype [30]. So, like in cortical basal progenitors, Rnd3 limits proliferation of SVZ progenitors, but the mechanisms involved are different.

2.1.2 Rnd functions in neuronal migration

Migration of cortical neurons

In addition to defects in cortical progenitors, the *in vivo* knockdown of *Rnd3* in the embryonic cortex perturbs the radial migration of projection neurons [31]. Briefly, after detachment from the ventricular surface, the nascent cortical neurons become bipolar and move radially away to the SVZ/intermediate zone (IZ), where they acquire a multipolar shape. Then, neurons enter into the cortical plate (CP) and become bipolar, extending a leading process (future apical dendrite) towards the pial surface and a trailing process (future axon) in the opposite direction. Upon multi to bipolar transition, neurons establish dynamic contacts with RGC fibres and subsequently use them as a scaffold to migrate to the upper part of the CP. During this phase, neurons use a mode of migration called locomotion, which is characterized by repetitive migratory cycles of extension of the leading process, translocation of the nucleus, and retraction of the trailing process.

After *Rnd3* silencing, neurons display an abnormal morphology and movement in the CP. Indeed, instead of being bipolar, *Rnd3* deficient neurons exhibit an enlarged leading process with numerous branches and the coupling between the nucleus and centrosome is perturbed, which altogether disrupt the coordinated movement of the locomoting neurons [8] (Figure 2a). *Rnd2* has been shown to be also important for this process of radial migration but this Rnd protein, in contrast to *Rnd3*, regulates the multipolar to bipolar transition [32–34] (Figure 2a). Indeed, *Rnd2*-deficient neurons display longer processes at the multipolar stage and fail to leave the IZ, which eventually leads to the accumulation of cells in the IZ of *Rnd2*-knocked down cortices and a concomitant reduction of neurons reaching the CP, in comparison to control cortices [8,33]. Despite these different functions, both *Rnd2* and *Rnd3* regulate neuronal migration by inhibiting RhoA, but only partially for *Rnd2*. The inhibitory effect of *Rnd3* on RhoA activity depends on its interactions with p190RhoGAP, whereas *Rnd2*'s RhoA inhibitory activity does not. Moreover, it has been demonstrated that Plexin B2 competes with p190RhoGAP for binding to *Rnd3*, thus blocking the *Rnd3*-mediated inhibition of RhoA, which allows a cell-extrinsic control of RhoA activity in migrating neurons [35]. In addition,

although both *Rnd2* and *Rnd3* regulate actin dynamics in migrating neurons, only *Rnd3* promotes neuronal migration by inhibiting RhoA-mediated actin polymerization and remodelling [8]. Interestingly, the distinct subcellular localization of *Rnd2* and *Rnd3* and the resultant modulation of RhoA activity in different cell compartments underlie the difference in their effects. *Rnd3* owes its distinct role in neuronal migration to its localization and interaction with RhoA at the plasma membrane. *Rnd2* is expressed in early endosomes [8] but the mechanisms by which *Rnd2* promotes cortical neuron migration and inhibits RhoA remains unknown. Two important factors that could mediate *Rnd2* activity in the embryonic cortex are the vacuolar protein sorting 1-A (Vps4-A) [7] that regulates endosome recycling and the proteins Bacurd1/Kctd13 and Bacurd2/Tnfaip1 that could reduce RhoA levels through their BTB-domain containing adaptors for Cul3-mediated RhoA degradation (Table 2). In particular, Bacurd1/Kctd13 and Bacurd2/Tnfaip1 have been shown to control long-term positioning of cortical neurons within the postnatal mouse cerebral cortex and thus their interaction with both *Rnd2* and *Rnd3* might be relevant to ensure correct neuronal migration [36].

Importantly, different transcription factors control radial migration in the developing cerebral cortex via *Rnd2* or *Rnd3* [31]. Indeed, the proneural transcription factor Neurogenin2 (Neurog2) has been shown to promote the migration of nascent cortical neurons by inducing the expression of *Rnd2* while another proneural factor, *Ascl1*, promotes neuronal migration in the cortex through direct regulation of *Rnd3* [8,33]. Neurog2 and *Ascl1* directly bind to E-box DNA sequences within enhancers located 3' to the *Rnd2* and *Rnd3* gene, respectively [8,33]. Other transcription factors such as RP58, Scratch2, and COUP-TFI promote cortical radial migration, but in contrast to Neurog2, by repressing *Rnd2* expression [37,38,41]. In particular, RP58 and Scratch2 regulate the same 3' enhancer as Neurog2, suggesting that the three factors might compete to fine-tune appropriate levels of *Rnd2* in the developing cortex [39,40,41].

Migration of neuroblasts in the rostral migratory stream

In addition to an excessive proliferation in the SVZ, cell migration through the RMS is impaired in mice lacking *Rnd3* expression [28] (Figure 2b). In this RMS, newly generated neuroblasts migrate tangentially in chains surrounded by astrocytes in a well-organized manner. In *Rnd3*-deficient mice, the migrating cells do not form a compact structure but instead are scattered and their

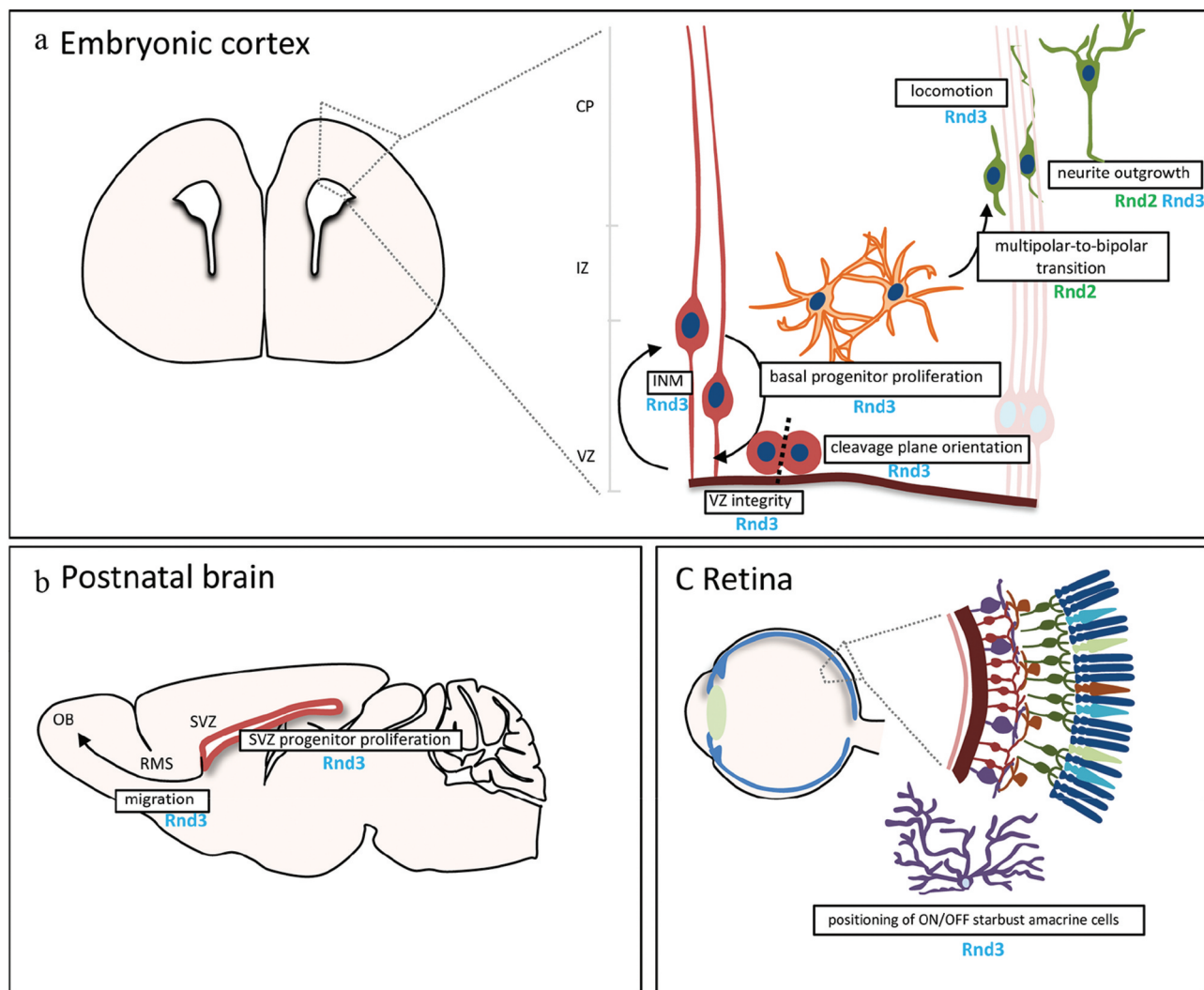


Figure 2. In vivo roles of Rnd proteins in the nervous system. A) Schematic of the murine embryonic cortex, showing the cellular events regulated by Rnd proteins. Neural stem cells (NSCs, in red) are attached to one another in the ventricular zone (VZ) by apically located adherens junctions. NSCs coordinate nuclear movement with the cell cycle in a process known as INM (interkinetic nuclear migration). Once NSCs start differentiating, they generate neuronal committed basal progenitors (in orange) that divide in the intermediate zone (IZ), away from the VZ. Basal progenitors and recently born neurons in this basal region exhibit multipolar morphology and transit to a bipolar shape (in green) before entering the cortical plate (CP) and migrate through locomotion to the pial surface. Nascent neurons establish their polarity while they migrate, with a trailing and a leading process that will finally develop into axon and dendrites, respectively. B) Scheme of the postnatal brain in a sagittal section. After proliferation of NSCs in the subventricular zone (SVZ) along the lateral ventricles, newborn neurons migrate to the olfactory bulb (OB) through the rostral migratory stream (RMS). C) Scheme of the eye and the retina, showing the different neuronal layers in the retina. Position of the amacrine cells (in purple) is regulated by Rnd3.

nucleus and cytoplasm are oriented in different directions [28]. Moreover, after BrdU injection, most of the BrdU-labelled cells in the mutant remain in the caudal RMS, closer to the SVZ, and only a small percentage arrives to the OB [28]. *In vitro*, neuroblasts from *Rnd3*-null SVZ-derived neurospheres migrate slower and cover lesser distances compared to control cells, which further suggests that Rnd3 is essential for the proper migration of neuroblasts in the RMS [28].

Migration of retinal interneurons

Recently, Rnd3 has been shown to be also crucial for correct positioning of retinal interneurons called starburst amacrine cells (SACs) [42] (Figure 2c). These neurons occur as pairs, with one member responding to increases (ON cells) and the other to decreases (OFF cells) in light intensity. SACs in the central mouse retina are born at the apical edge of the retinal

neuroblast layer from around embryonic day (E) 10 to E14. They then migrate basally to reach the border of the outer and inner neuroblast layers (ONBL, INBL). After E16, about half continue basally to the INBL, where they become ON SACs; the others remain in the ONBL and become OFF SACs. SACs are fully integrated into direction-selective circuits by the time of eye-opening at P14. Interestingly, *Rnd3* is selectively expressed by OFF SACs and regulates their migration but is repressed by *Fezf1* in ON SACs, allowing their entry into the INBL [42]. *Rnd3* acts thus to enable distinct positioning of ON and OFF SACs in the developing retina (Figure 2c) but the mode of action of *Rnd3* in these cells remains to be elucidated.

2.1.3 *Rnd* functions in neuronal polarization

A mature neuron typically has several dendrites that receive inputs from presynaptic neurons and one axon that relays information to post-synaptic neurons. The development of these specialized structures is preceded by the formation of neurites, which are small protrusions sprouting from the cell body and lacking molecular and structural characteristics of mature axonal or dendritic processes, such as the expression of Tau or Microtubule-associated protein 2 (MAP2) respectively.

Neurite outgrowth

The three *Rnd* members have been implicated in the regulation of neurite outgrowth and this has been mainly demonstrated *in vitro* in PC12 cells. These cells, derived from a rat pheochromocytoma, have been used extensively over the years to study neuronal differentiation because they exhibit a typical phenotype of neuronal cells sending out neurites in response to nerve growth factor (NGF) [43] or basic fibroblast growth factor (bFGF) [44]. Forced expression of *Rnd1* or *Rnd3* in these cells, in the absence of growth factors, causes the formation of many neuritic processes [45–47] while their knockdown suppresses the FGF or NGF-induced neurite outgrowth, respectively, [47,48]. Both *Rnd1* and *Rnd3* stimulate neurite outgrowth in PC12 cells through inhibition of RhoA signalling [47,48]. More precisely, in the resting state, *Rnd1* interacts with FRS2 β (Table 2), a docking protein of the fibroblast growth factor (FGF) receptor-1, and this prevents the interaction of *Rnd1* with other molecules [48]. In the presence of FGF, the receptor FGFR1 is activated and phosphorylates FRS2 β . This phosphorylation releases *Rnd1* and the liberated *Rnd1* then inhibits RhoA activity [48]. *Rnd1* thus plays an important role in the FGF signalling during neurite development in PC12 cells and its activity is regulated by FGF receptor

through FRS2 β . Moreover, the Stress-Inducible Protein-1 (STI1), a co-chaperone that associates directly with Hsp70/Hsp90 heat shock proteins and modulates their chaperone activities has been shown to specifically bind to *Rnd1* and regulates its activity in PC12 cells [46] (Table 2). Regarding *Rnd3*, similar results have been obtained in cultured hippocampal neurons from mice lacking *Rnd3* expression [49]. Indeed, *Rnd3*-deficient neurons exhibit a decrease in neurite outgrowth and the RhoA/ROCK pathway, like in PC12 cells [47], is involved in this neuronal alteration [49].

In contrast to *Rnd1* and *Rnd3*, *Rnd2* induces inhibition of NGF-induced neurite outgrowth in PC12 cells and stimulates RhoA activity through Pragmin (Table 2), a catalytically inactive pseudo-kinase, although there is no direct biochemical connection between Pragmin and RhoA [50]. Moreover, *Rnd2* stimulates neurite branching in these cells through Rapostlin [51], a molecule involved in the formation of endocytic vesicles [52]. Rapostlin, also named FBP17, binds to *Rnd2* through an HR1 domain, related to those identified in RhoA/Rac1 effectors such as PRK/PKN. It acts through the ability of its F-BAR domain to promote membrane invagination [53]. *Rnd2* may be thus involved in the regulation of different aspects of neuronal morphology development through different effectors, such as neurite branching with Rapostlin and neurite outgrowth inhibition with Pragmin.

Although the regulation of neurite outgrowth by *Rnd* proteins has been studied extensively *in vitro*, the control of the corresponding developmental process *in vivo* has been poorly addressed. Only very few studies have looked at this aspect in the developing cortex for *Rnd2* and *Rnd3*. *Rnd3*-silenced neurons that reach the CP present multiple thin processes extending from the cell body and the leading process suggesting that *Rnd3* knockdown in this system promotes neurite outgrowth, which is in contrast with the *in vitro* data previously described [47]. Moreover, *Rnd2* deficient neurons exhibit more and longer processes in the IZ [8,33] while its overexpression results in the disappearance of neurites and in the rounding of IZ cortical neurons [33]. *Rnd2* and *Rnd3* are thus required for the proper initiation of polarization in nascent pyramidal cortical neurons, an essential step for their concomitant migration.

Axon and dendrite development

The development of the axon and dendrites has been studied extensively in rat primary hippocampal cultures and described as follows [54,55]. After 1.5–3 days *in vitro*, one of the neurites elongates rapidly without

retraction to form the axon and several days later (days 4–7) the remaining neurites grow at a slower rate to form the dendrites. Lastly, the axon develops further and dendrites continue to branch. Using this model, it has been shown that *Rnd1* knockdown suppresses axon formation whereas its overexpression induces the generation of multiple axons [56]. Moreover, the loss of the microtubule-destabilizing protein Superior Cervical Ganglion10 (SCG10) blocks the formation of axons after *Rnd1* overexpression, indicating that SCG10 acts downstream of *Rnd1*. Interestingly, *Rnd1* interacts directly with SCG10 and enhances its activity (Table 2), which suggests that *Rnd1* regulates axon extension by modulating microtubule dynamics through SCG10 [56]. Besides this role, *Rnd1* also controls growth cone collapse in the developing axons of hippocampal neurons, an essential step for correct targeting of growing axons [57]. The growth cones, located at the tip of the growing axons, respond to attractive and repulsive cues in the local environment to guide the axons towards their specific targets. Among these cues, the semaphorins were originally identified as repulsive axonal guidance molecules and their function is mediated by plexins, which are classified into four subfamilies: Plexin-A1–4, Plexin-B1–3, Plexin-C1, and Plexin-D1 [58]. Interestingly, *Rnd1* directly interacts with the cytoplasmic domain of Plexin-B1 [59] (Table 2) and the Plexin-B1-*Rnd1* complex stimulates the GTPase activity of R-Ras, inducing growth cone collapse in hippocampal neurons in response to semaphorin 4D (Sema4D) and thus repulsion [57]. *Rnd1* also binds to Plexin-A1 [60] and is required for the Sema3A-Plexin-A1-mediated repulsion but this has been demonstrated in COS-7 cells, so not in a neuronal context [61].

In addition to *Rnd1*, *Rnd2* and *Rnd3* are also critical for axon development, especially for its outgrowth. Indeed, *Rnd3*-deficient hippocampal neurons display shorter axons compared to control neurons and this action seems to depend on the RhoA/ROCK signalling [49]. Regarding *Rnd2*, its action involves the plexin-D1 receptor. Indeed, *Rnd2* binds to Plexin-D1 in cortical neurons (Table 2) and this interaction is required for Sema3E/Plexin-D1-induced inhibition of axon outgrowth in these neurons [62].

Regarding dendritogenesis, although the role of the RhoGTPases and in particular the functions of RhoA, Rac1 and Cdc42 have been extensively characterized [63], only one study has described an involvement of *Rnd1* in the regulation of this process [64]. In this study, overexpression of *Rnd1* stimulates dendritic growth and branching in cultured hippocampal neurons. Inversely suppression of endogenous *Rnd1* expression by RNA interference abolishes dendritic

development promoted by KCl, an agent that mimics the depolarizing effects of persistent neuronal activity, or by brain-derived neurotrophic factor (BDNF) [64]. However, in the absence of these treatments, the knockdown of *Rnd1* does not affect significantly the dendrites of the cultured neurons suggesting that *Rnd1* is involved in signalling pathways of neuronal activity-dependent dendritic development [64]. Moreover, like for neurite outgrowth, the action of *Rnd1* seems to be linked to an inhibition of the Rho/ROCK pathway [64]. While a direct role for *Rnd2* and *Rnd3* in dendritogenesis is missing, it is important to mention that their interacting partners Bacurd1/Kctd13 and Bacurd2/Tnfrsf1 play an essential role in dendritic maturation of cerebral cortical neurons [36]. Moreover, the activity of Bacurd1/Kctd13 in synaptic transmission partially works via RhoA regulation [65], thus reinforcing potential *Rnd* involvement in the pathway.

2.1.4 *Rnd* functions in spine formation

The final step in the development of a neuron is the formation of spines. These small dendritic protrusions contain receptors and other proteins necessary for synaptic transmission, and thus allow communication with appropriate targets into neural circuits.

The three members of the *Rnd* family have been shown to control spine morphogenesis, at least *in vitro*. In primary hippocampal neurons, *Rnd1* is strongly localized in spines [66]. Ectopical overexpression of *Rnd1* promotes the elongation of dendritic spines, whereas its silencing causes an increase in the percentage of headless protrusions accompanied by a reduction in spine number [66]. Knockdown of *Rnd2* also reduces spine density in cultured hippocampal neurons [53]. *Rnd1* and *Rnd2* are thus critical for normal spine formation but the downstream mechanisms remain to be understood. Similarly, suppression of *Rnd3* by shRNA constructs reduces dendritic spine density and its overexpression has an opposite effect [67]. Moreover, knockdown of *Rnd3* completely blocks BDNF-stimulated spine formation in cultured hippocampal neurons indicating that *Rnd3* is crucial for BDNF-dependent spinogenesis [67]. Interestingly, these changes in the number of dendritic spines appear to reflect changes in the number of functional synapses, as the number of miniature excitatory post-synaptic currents (mEPSCs) correlates with changes in spine number. Indeed, expression of *Rnd3* increases the frequency of mEPSCs approximately twofold over control, and targeted knockdown of *Rnd3* modestly reduces mEPSC frequency, and significantly decreases the effect of BDNF on mEPSC frequency [67]. Finally, *Rnd3*

appears to regulate spinogenesis via p190RhoGAP regulation of RhoA, as its effect on spines is blocked by expression of a constitutively active mutant of RhoA or suppression of p190RhoGAP [67].

Taken together, these studies demonstrate that the three members of the Rnd family play multiple and crucial functions during neuronal development (Table 3). However, future studies are needed to better characterize these functions *in vivo* and address their roles in different brain regions at specific developmental stages.

2.2 In pathological conditions

As a consequence of their important physiological role in neuronal development, alterations in Rnd protein expression could contribute to the pathogenesis of many brain disorders. Perturbations in Rnd expression have been described in various forms of cortical malformations and other pathological events. However, so far, there is no direct evidence to support a role for these proteins in the aetiology and progression of these diseases.

Upregulation of Rnd2 expression has been found in brains of epileptic patients [68] and in patients with focal cortical dysplasia (FCD), who also suffer from drug-resistance epileptic seizures [69]. The brain of FCD patients exhibits various gross malformations, such as abnormal gyrification, thickened cortex and local areas of subcortical neurons, which can be attributed to altered neuronal generation and migration. Recent genetic studies of FCD patients revealed a decrease in certain microRNAs, one of which targets the proneural transcription factor *Neurog2*, which is known to control *Rnd2* expression in the embryonic cortex [33]. Thus, when the levels of this miRNA (mir-34a) decrease, *Neurog2* and its transcriptional target *Rnd2* are upregulated [69]. Considering the crucial role that Rnd2 plays in neuronal migration in the cortex, it is possible that variations in *Rnd2* levels observed in these patients contribute to the aetiology of cortical malformations.

Abnormal expression levels of Rnd3 are also thought to be involved in cortical malformations, although direct evidence is still missing. *Rnd3* is one of the genes upregulated in human brain organoids derived from patients with periventricular heterotopia (PH), a malformation characterized by the accumulation of newborn neurons close to lateral ventricles [70]. These results echo the block in migration observed upon Rnd3 overexpression in the embryonic murine cortex [8]. However, how *Rnd3* upregulation might be controlling neuronal stalling is not known. It is possible

that Rnd3 regulation of RhoA activity, as shown in neurogenesis [8,35], might be involved. Indeed, Rnd3 acts via inhibiting RhoA in nascent cortical neurons and, since *RhoA* deletion in mice causes cortical malformations similar to human subcortical band heterotopia [71,72], it is possible that dysregulation of *Rnd3* expression might lead to cortical malformations. Further studies are needed to directly assess this hypothesis.

Additional indirect evidence suggesting that Rnd3 might play important roles in human brain disorders comes from the analysis of *Rnd3* knockout animals. Indeed, mice lacking *Rnd3* die shortly after birth and exhibit neuromotor impairment and neuromuscular alterations, such as the loss of the peroneal nerve and reduction in the number of spinal motor neurons, which can be attributed to abnormal development and maturation of the nervous system [73]. In another model of *Rnd3* knockout animals, the genetic deletion of *Rnd3* induces an excessive proliferation of the ependymal cells, which form the epithelial lining of the brain ventricles [18]. This effect, which is due to an up-regulation of Notch signalling in these cells, blocks the cerebral aqueducts and leads to the development of hydrocephalus [18]. Moreover, *Rnd3* knockout animals exhibit an enlarged and disorganized postnatal SVZ, which is due to increased NSC proliferation [28,30]. Since the SVZ contains neural stem and progenitor cells that produce new neurons over the course of adult life, aberrant SVZ neurogenesis might lead to long-term effect on adult neurogenesis and eventually contribute to mental disorders and/or neurodegenerative neurological disorders.

A fundamental process contributing to neurodegeneration is neuroinflammation, which can lead to neuronal cell death in diseases such as Parkinson's and Alzheimer's. Importantly, Rnd3 has been shown to promote the expression of the pro-survival factor BDNF by microglia cells, the immune cell compartment of the nervous system, and to exert in this way a neuroprotective role [74]. Moreover, the expression of another Rnd protein, *Rnd1*, has been found to be increased in the hippocampus of 2 out of 7 Alzheimer patients, in comparison to healthy controls [75]. Altogether these works indicate a potential role for Rnd proteins in neurodegenerative disorders. However, whether Rnd1 and Rnd3 play an active role in neurodegeneration and contribute to the formation of the pathological features or whether Rnd expression in this context represents a neuroprotective response is currently unknown. Future work should focus on the role of Rnd proteins in neurodegeneration in order to provide more mechanistic insights into these

degenerative disorders that affect millions of people worldwide.

3. Rnd proteins in the vascular system

Rnd proteins are expressed in various cells of the vascular bed, such as endothelial cells and smooth muscle cells. As contractility is a key mechanism for the performance of the vascular system, many studies addressed the involvement of the RhoA/ROCK pathway and Rnd proteins in this context. Rnd1 and Rnd3 were described to be involved in physiological conditions, whereas the majority of studies carried out to date on the role of Rnd protein family in vascular diseases mainly focused on Rnd3.

3.1 In physiological conditions

Rnd1 expression was found modulated upon various stimuli in endothelial cells, even if the pathways are not always deciphered. The vascular endothelial growth factor (VEGF) is a key regulator of endothelial cell migration, proliferation, and inflammation, which leads to activation of several signalling cascades. The expression of Rnd1 was found positively regulated by the NFATc1 (nuclear factor of activated T cells) transcription factor in VEGF-stimulated primary-cultured endothelial cells [22]. Silencing of *RND1* impaired vascular barrier function, caused RhoA hyperactivation, and further stimulated VEGF-mediated vascular outgrowth from aortic rings [22]. Thus, Rnd1 is one of the downstream targets of VEGF involved in endothelial cell activation. An upregulation of Rnd1 expression was also observed in human microvascular endothelial cells (HMVEC) after interleukin IL-1 β treatment [76] and in mouse heart-isolated endothelial cells upon lipopolysaccharide (LPS) stimulation [77] suggesting that Rnd1 may be involved in vascular inflammation.

Rnd1 was found specifically expressed in human saphenous vein smooth muscle [78]. Vascular smooth muscle contractility is primarily regulated by the level of phosphorylation of myosin light chain. Beside RhoA/ROCK playing major roles in the Ca²⁺ sensitization through the phosphorylation and inhibition of the subunit of MLCP (Myosin light chain phosphatase), Rnd proteins were also implicated in muscle contraction by modification of their expression level. In smooth muscle, it has been showed that Rnd1 inhibits Ca²⁺ sensitization and interferes with the RhoA-dependent mechanism. Moreover, the sex hormone steroids such as oestradiol, known to induce the contractility of vascular smooth muscles increase the expression of Rnd1 [79], Rnd2 and Rnd3 [80] in muscles and decrease

agonist-induced Ca²⁺ sensitization. Interestingly, large transcriptome studies using DNA array technology and RT-qPCR analyses found Rnd1 [81], Rnd2 and Rnd3 [82,83] upregulated in animal myometrium contractility during pregnancy indicating that they may indeed play an important role via the regulation of RhoA/Ca²⁺ sensitization.

Recent emerging evidence shows the important physiological role of Rnd1 and Rnd3 in animal heart. Rnd1 was recently shown to participate in the cardiac remodelling in response to mechanical or humoral stress [84]. Rnd1 was indeed discovered as one of the significantly upregulated genes in rat ventricular cardiomyocytes upon dynamic mechanical stretch or phenylephrine stimulation or in mice after pressure overload induced by transverse aortic constriction. Rnd1 overexpression induces cardiomyocyte proliferation and hypertrophy. Thus, Rnd1 is described as a sensor of biomechanical stress in the heart [84]. Using loss-of-function approaches, *Rnd3* gene was found indispensable for mouse development and for the intracellular calcium homeostasis regulation in cardiomyocytes. Indeed, *Rnd3*-null mice died at the embryonic stage with foetal heart arrhythmias [85]. This phenotype is due to destabilizing ryanodine receptor type 2 channels, contributing to abnormal intracellular Ca²⁺ release. *Rnd3* deficiency mediates the activation of kinase PKA signalling leading to destabilizing ryanodine receptor type 2 by hyperphosphorylation. The same results were obtained with different cell types either after Rnd3 inhibition in neonatal rat cardiomyocytes, HEK293T epithelial cells and C2C12 myoblasts or in fibroblasts isolated from *Rnd3*^{-/-} mice. The regulatory role of Rnd3 is not only important in an embryonic heart, but also in adult. Indeed, the cardiomyocytes isolated from adult *Rnd3* \pm mouse hearts display significantly higher calcium transient frequency compared to the control cells, a phenotype that is attenuated by PKA inhibitor. Mechanistically, it has been shown that Rnd3 is a regulator of the beta2-adrenergic receptor (β 2AR). Thus, the lack of Rnd3 attenuates the ubiquitinylation of β 2AR, resulting in the accumulation of β 2AR protein, which promotes the activation of PKA [85].

3.2 In pathological conditions

Several studies suggest a link between Rnd3 and the progression of vascular diseases. Rnd3 can serve as a potential therapeutic tool to control the endothelial barrier integrity under certain inflammatory conditions. It is detected in both the rat mesenteric microcirculation and in cultured HUVEC endothelial cells.

The ectopic expression of Rnd3 in endothelial cells challenged with an inflammatory stimulus such as thrombin promotes enhanced barrier function. This effect reduces the thrombin-induced changes in the actin cytoskeleton and prevents cellular retraction. Moreover, Rnd3 delivered in the mesenteric venules isolated from rats following haemorrhagic shock reduces the microvascular permeability [86].

The involvement of Rnd3 in the control of endothelial barrier function adds to the growing list of Rnd3 functions, including Ca^{2+} homeostasis in the heart and a potential role in cardioprotection against heart failure. Three studies from the Chang laboratory explored the involvement of Rnd3 in cardiomyopathies. They revealed a significant decrease of *Rnd3* mRNA levels in human failing hearts [87]. To investigate the biological function of Rnd3 in heart failure, they established a patient-relevant mouse model, *Rnd3* \pm haploinsufficient mice. The *Rnd3* \pm haploinsufficient mice are viable and survived into adulthood without detectable abnormalities in heart morphology compared to the wild-type animal under physiological conditions. However, these mice developed heart failure after haemodynamic stress by transverse aortic constriction challenge. This abnormality is associated with an elevated ROCK activity. Treatment with Fasudil, the only clinically approved ROCK inhibitor, attenuates the apoptotic cardiomyopathy observed in *Rnd3* \pm mice and improves cardiac functions [88]. The same group further demonstrates that Rnd3 deficiency resulted in an angiogenesis defect in the mouse heart in response to pressure overload. Thus, they demonstrated that Rnd3 acts as a pro-angiogenic factor involved in the cardiac responsive angiogenesis [87]. In a more recent study, Rnd3 was found to protect the heart from the inflammatory response during the cardiac healing process after myocardial infarction (MI) [89]. Indeed, Rnd3 expression is rapidly increased during the acute inflammatory phase in animal heart after cardiac ischaemia. The use of cardiomyocyte-specific Rnd3 heterozygous mice revealed an excessive inflammatory response with deleterious cardiac function after MI. Mechanistically, Rnd3 negatively regulates the proinflammatory gene expression programme by directly blocking the key inflammatory regulator NF- κ B nuclear translocation. Rnd3 is able to interact with the NF- κ B components p50 and p65 in the cytosol, interrupting p65/p50 heterodimerization (Table 2). Manipulation of Rnd3 could serve as a potential therapeutic approach as the authors found that patients with a higher expression level of Rnd3 exhibit a better prognosis in cardiac function recovery [89].

Primary studies showed links between Rnd3 and the progression of cardiovascular diseases. The stress hormone urocortin is known to confer significant protection against myocardial ischaemia/reperfusion (I/R) injury. Microarray analysis showed an upregulation of Rnd3 in rat infused with urocortin before the onset of reperfusion [90]. However, the involvement of Rnd3 in urocortin-mediated cardioprotection remains to be determined. Interestingly, studies have evidenced that urocortin is also key at the end of the pregnancy period as low level of urocortin is required to provoke an increase in the contractility of the uterus [91]. Whether a decrease of Rnd3 expression is also required in this context has not been explored. But, for the maintenance of successful pregnancy, fusion of placental villous cytotrophoblasts with the overlying syncytiotrophoblast is essential, and disturbances in this process have been implicated in pathological conditions such as pre-eclampsia (PE). Rnd3 expression was found to promote the fusion of BeWO choriocarcinoma cells, a model of villous cytotrophoblast fusion [92]. Moreover, Johnson & collaborators defined the UTR-3 RND3 SNP (rs115015150) as a risk factor for the development of maternal cardiovascular disease and pre-eclampsia [93]. PE is a pregnancy-specific syndrome, characterized by maternal new-onset hypertension and proteinuria by an abnormal placental function due to trophoblast invasion and failure of spiral artery remodelling. Interestingly, Rnd3 expression levels were found elevated in PE placental tissues [94]. This increase of Rnd3 expression was attributed to the decrease of two Rnd3-targeting long non-coding (lnc) RNAs, TUG1 and HOXA11-AS, levels in PE [94,95]. In addition, Li and collaborators determined that the lncRNA HOTTIP expression is lower in PE placenta than in normal tissue, causing also the overexpression of Rnd3 [96]. Functional *in vitro* studies further determined that an upregulation of Rnd3 inhibits trophoblast cell proliferation and migration [94–96]. Thus, Rnd3 appears as a key protein involved in the progression of PE. The expression of Rnd3 in PE may also be regulated by miRNAs. However, in sharp contrast, Rnd3 expression was described as a target of miR-182-5p found highly expressed in placenta of PE patients [97]. The overexpression of miR-182-5p induces the invasion and migration of trophoblasts by regulation of Rnd3 expression. Indeed, Rnd3 expression reversed the effects exerted by the overexpression of miR-182-5p [97]. Overall, these results suggest that Rnd3 is an important factor in PE disease.

4. Rnd proteins in cancers

Deregulation of Rnd proteins is associated with cancer development and progression. The role of Rnd1 and Rnd3 in oncogenesis has been the subject of numerous studies, while Rnd2 function has been less explored. As the role of Rnd1 and Rnd3 in cancers has been extensively discussed elsewhere, we refer the reader to two recent reviews [20,98]. Here, we summarize the degree of altered expression and the roles of all three Rnd family members in human malignancies. We made use of the large number of cancer genome-sequencing data to compare Rnd1, Rnd2 and Rnd3 involvement in cancers.

4.1 Rnd genetic alteration in cancers

Although a few cancer genes, such as *RAS*, are mutated in a high proportion of tumours of a given type (>20%), most are mutated at intermediate frequencies (2–20%). *RHOA* and *RAC1* are part of this latter category. Genetic alterations, including gene amplification, deletion or fusion, and missense or truncating mutations, for *RND1*, *RND2* and *RND3* genes are rather rare events in tumours. Indeed, data from the Cancer Genome Atlas (TCGA) PanCancer Atlas (querying 10,967 human samples in 32 studies) extracted from the CBioPortal database show genetic alteration in 0.9% of the samples for each *RND1*, *RND2* or *RND3* gene (Figure 3). As most of these alterations are mutually exclusive, this raises to about 2.7% for all three genes (290 of queried samples). Gene fusions were found in only two samples in this cohort: *DDX23-RND1* fusion in hepatocellular carcinoma and *MRC2-RND2* fusion in glioblastoma multiforme. Locus deletion events were reported to be responsible for down-regulation of RND expression in few tumour types. The 12q12-q13 region, which comprises the *RND1* gene, is deleted in several cancers, such as adenoid cystic carcinoma [99], pancreas cancer [100] and breast cancer [21]. In most of the cases these deletions are heterozygous, requiring additional mechanisms to totally inactivate *RND1* expression in tumours. Again, less is known about *RND2*. Of interest, *RND2* human gene is the centromeric neighbour of the breast-ovarian cancer susceptibility gene *BRCA1* on chromosome 17. One study describes the first case of a patient, with no strong family history of the disease, who developed early-onset bilateral breast cancer with a *de novo* complete *BRCA1* gene deletion in the germinal line [101]. Even if the large genomic rearrangement detected removed the *RND2* gene, the involvement of Rnd2 in the onset of the disease remains to be studied. In addition, data

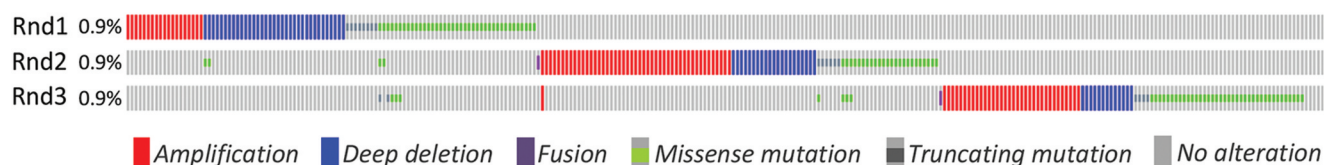
from the TCGA PanCancer Atlas revealed that deep deletion of *RND2* gene are also found in adrenocortical carcinomas, prostate adenocarcinoma, uveal melanoma and ovarian epithelial tumours, even if no study so far focused on their implication (Figure 3). Even if deep deletions for the *RND3* gene were also found in global approaches (Figure 3), in prostate adenocarcinoma, so far, no study confirmed these events. Similarly, amplifications that may lead to up-regulation of *RND* expression are detected in few cancer types (Figure 3). In non-small cell lung cancer, overexpression of *RND3* expression was significantly associated with the patients' smoking history and DNA copy number changes [102].

Somatic mutations, either missense or truncating mutations, are found at a frequency of 0.5%, 0.3% and 0.4% for respectively *RND1*, *RND2* and *RND3* genes in this dataset. Missense mutations (in green on Figure 3) were found all along the coding sequence, without hot spots. The impact of most of these mutations on their respective proteins is currently unknown. Available data concern only *RND1*; three missense mutations, which replaced evolutionarily conserved amino acids and found in a cohort of 96 breast cancers, were characterized [21]. Unlike wild-type Rnd1, G70R, E98D, and F180C mutants expressed in MCF-10A cells are not able to inhibit tumour cell growth. Moreover, they are affected in their subcellular localization and in their ability to bind to plexin B1 to inhibit the downstream Raf-Erk cascade [21]. Thus, these mutations participate to repress Rnd1 properties in mammary tumour cells. Even if *RND1* point mutations are rare in tumours, this study suggests that in combination with gene deletion or epigenetic silencing they may inactivate *RND1* in human breast cancer and favour breast tumour initiation and progression through activation of Ras-MAPK signalling.

4.2 Alteration of Rnd expression in cancers

The most frequent alterations found in tumours remain deregulation of mRNA or protein expression. Thanks to the fact that Rnd is deficient in GTPase activity, compared to other Rho members, an alteration of their expression reflects more an alteration of their activity. We used the GEPIA (Gene Expression Profiling Interactive Analysis) web server, a valuable and highly cited resource, to analyse *RND1*, *RND2* and *RND3* gene expression in tumour and normal samples [103]. This publicly accessible web interface (<http://gepia2.cancer-pku.cn/>) compiles RNA sequencing data from the TCGA and the GTEx projects. Figure 4 presents the expression of RND transcripts in 32 different types of cancer and shows that,

a -



b-

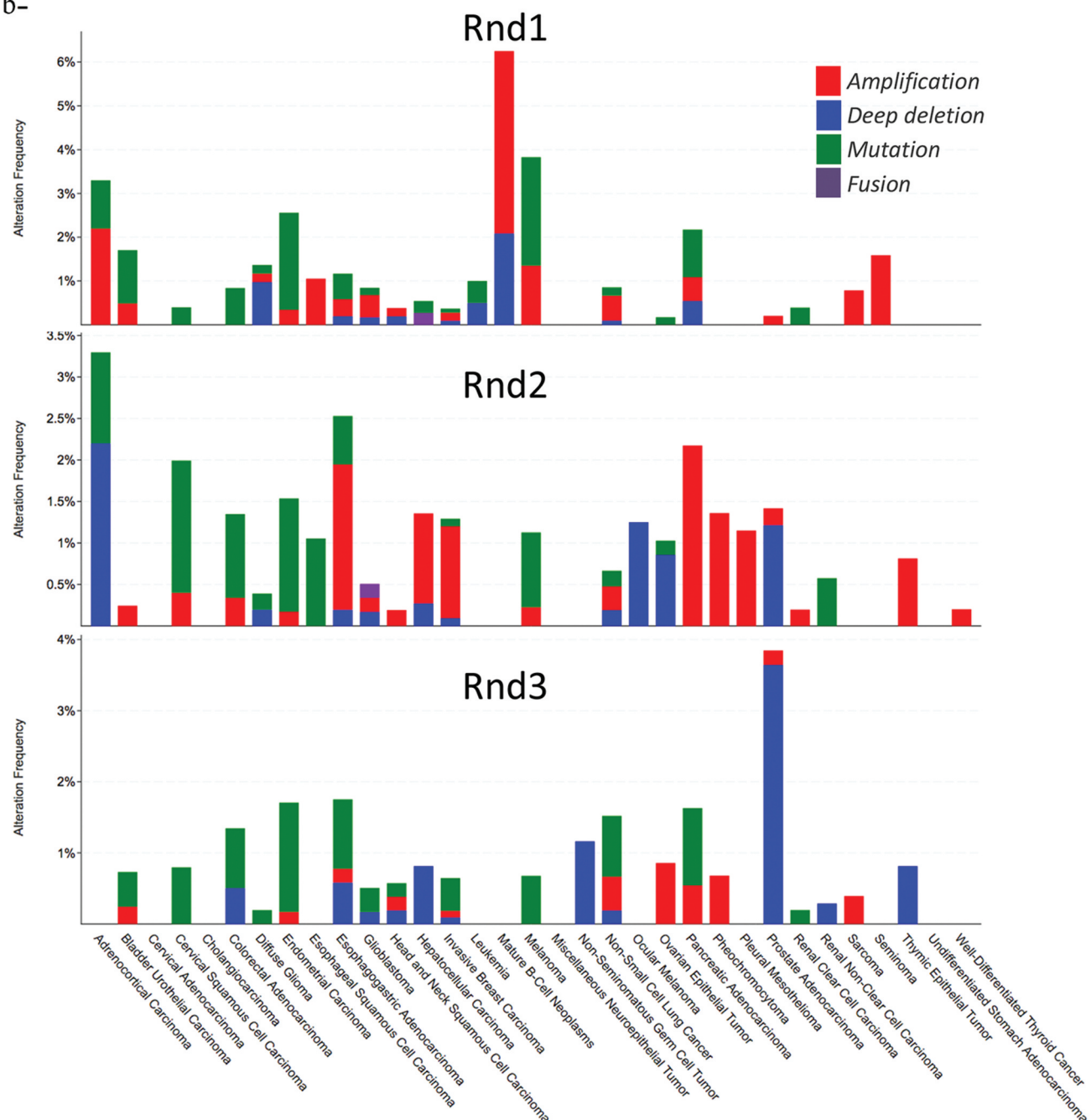


Figure 3. Genetic alterations of *RND* genes in cancers. Data extracted from the CBioPortal database (<https://www.cbioportal.org/>). Data from TCGA PanCancer Atlas studies pooling 32 studies and 10,967 samples were analysed for *RND1*, *RND2* and *RND3* genes. (a) Shown is the OncoPrint. Note that only tumours with *RND* alterations ($n = 290$) are present on the schema. (b) Shown is the Cancer Types Summary. The y axis shows the frequency of alterations, whereas the x axis indicates tumour types.

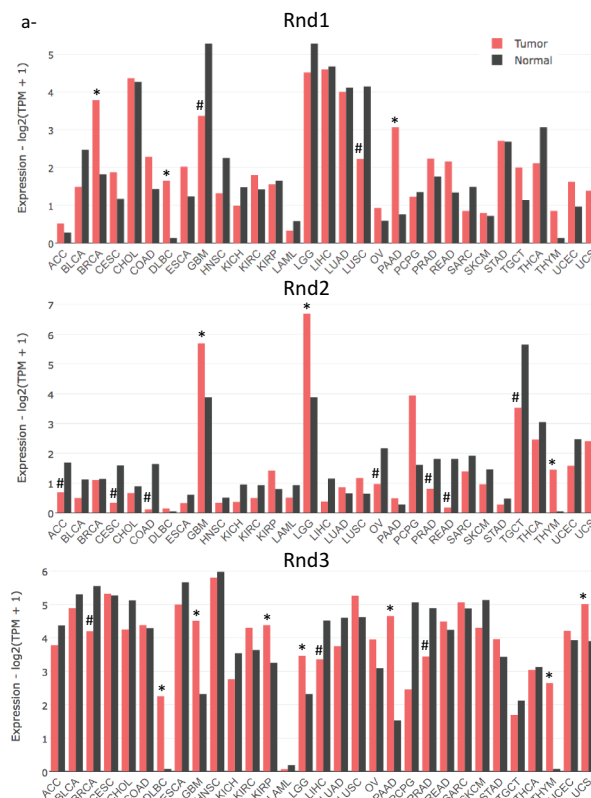


Figure 4. Expression data of *RND* genes in cancers. Data extracted from the GEPIA2 (Gene expression Profiling Interactive Analysis) database (<http://gepia2.cancer-pku.cn/>). Data from TCGA PanCancer Atlas studies pooling 32 studies and 10,967 samples were analysed for *RND1*, *RND2* and *RND3* genes. (a) Bar plot indicating the gene expression profile across all tumour samples (red bars) and paired normal tissues. The height of bar represents the median expression of certain tumour type or normal tissue (black bars). Tumours with a significant increase or decrease of Rnd expression are marked by an asteric (*) or a dièze (#), respectively. (b) Dot plot indicating the gene expression profile across indicated tumour samples (red dots) and paired normal tissues (green dots). Each dot represents expression of samples. Only tumour types with either up- or down-regulated Rnd gene expression are indicated. Tumour types: ACC, Adrenocortical carcinoma; BLCA, Bladder Urothelial Carcinoma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangio carcinoma; COAD, Colon adenocarcinoma; DLBC, Lymphoid Neoplasm Diffuse, Large B-cell Lymphoma; ESCA, Oesophageal carcinoma; GBML, Glioblastoma multiforme; HNSC, Head and Neck squamous cell carcinoma; KICH, Kidney Chromophobe; KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma; LAML, Acute Myeloid Leukaemia; LGG, Brain Lower Grade Glioma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; MESO, Mesothelioma OV Ovarian serous cystadenocarcinoma; PAAD, Pancreatic adenocarcinoma; PCPG, Pheochromocytoma and Paraganglioma; PRAD, Prostate adenocarcinoma; READ, Rectum adenocarcinoma; SARC, Sarcoma; SKCM, Skin Cutaneous Melanoma; STAD, Stomach adenocarcinoma; TGCT, Testicular Germ Cell Tumours; THCA, Thyroid carcinoma; THYM, Thymoma; UCEC, Uterine Corpus Endometrial Carcinoma; UCS, Uterine Carcinosarcoma; UVM, Uveal Melanoma.

depending on cancer types, *RND* expression is either down- or up-regulated.

Using this global approach, *RND1* is found significantly overexpressed in breast invasive carcinoma, diffuse large B-cell lymphoma and pancreatic adenocarcinoma. In addition, one study described the upregulation of *RND1* transcripts in oesophageal squamous cell carcinoma, where Rnd1 expression promotes the growth and the migration of cancer cells [104]. In another study on breast cancer, Rnd1 was found to behave as a tumour suppressor, in contrast with the TCGA data, highlighting the heterogeneity of breast carcinoma. Indeed, in mammary

epithelium, *RND1* loss was shown to activate Ras and the downstream Raf-Erk pathway, leading to uncontrolled proliferation and disruption of epithelial adhesion [21]. In mice, inactivation of Rnd1 in mammary epithelial cells induced highly undifferentiated and invasive tumours. Thus, genetic and epigenetic inactivation of *RND1* was proposed to underlie activation of the Ras-ERK pathway in a subset of breast cancers and participate in tumour initiation and progression [21]. *RND1* expression was also found significantly down-regulated in glioblastoma and lung squamous cell carcinoma (Figure 4). Interestingly, *RND1* downregulation was found to favour migration

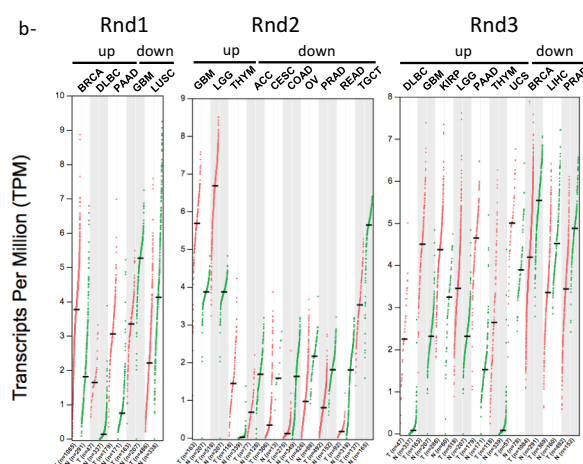


Figure 4. (countiued)

of glioblastoma stem-like cells derived from human samples [105]. In patients, low levels of *RND1* were shown to correlate with a decreased overall survival. Moreover, an *RND1*^{low} signature of six genes was proposed as an independent prognostic factor in glioblastoma [105]. Low expression of *RND1* was also associated with a bad prognosis for hepatocellular carcinoma (HCC) patients. Indeed, two studies described *RND1* as a tumour suppressor in liver cancers [106,107]. *RND1* expression was found decreasing as HCC progressed, with a lower expression in advanced stages. *In vitro*, *RND1* knockdown enhanced the malignant characteristics of HCC (Huh7 and PLC/PRF/5) cells, such as proliferation, invasion, and chemoresistance [106]. Thus, *RND1* was proposed as a novel biomarker for predicting prognosis of HCC patients.

Finally, recent data suggest that *RND1* is involved in response to anticancer agents. *RND1* expression was found rapidly induced upon treatment with the selective topoisomerase I inhibitor, camptothecin, in various cancer cells, including human osteosarcoma (U2OS), glioblastoma (U87), and colon carcinoma (HCT116) cells, and murine melanoma (B16F10) [108]. As shown for osteosarcoma cells, *RND1* expression protects them against drug-induced apoptosis, and thus, favours cellular resistance to topoisomerase I inhibitors [108]. This response to cancer therapeutics is not restricted to camptothecin, as *RND1* expression was also linked to cisplatin and sorafenib resistance in HCC patients [106,107]. Moreover, by analysing the correlation between *RND1* expression and the cellular response to 20 anticancer agents with the CCLE database (<https://portals.broadinstitute.org/ccle>), Mouly

and collaborators [20] suggest a role for *RND1* expression in the sensitivity of lung tumours to EGFR signalling pathway inhibitors.

RNA sequencing data revealed that *RND2* expression is up-regulated in brain tumours, either low-grade (brain low-grade glioma) or high-grade (glioblastoma) and in thymoma (Figure 4). On the contrary, it is down-regulated in adrenocortical carcinoma, and in colon and rectum adenocarcinomas. Moreover, in men, *RND2* expression, which is normally high in normal testis tissue, is found strongly decreased in testicular germ cell tumours and in prostate adenocarcinoma. In women, it is also down-regulated in cervical and ovarian carcinomas. So far, no study reported the impact of these deregulations of *RND2* on tumour development and progression. Thus, these data obtained from global approaches remain to be confirmed by further studies.

From GEPIA data, *RND3* expression is significantly overexpressed in seven types of tumours including diffuse large B-cell lymphoma, low- and high-grade brain tumours (i.e. low-grade glioma and glioblastoma), kidney renal papillary cell carcinoma, pancreatic adenocarcinoma, thymoma and uterine carcinosarcoma. Most of these up-regulations remain to be confirmed by molecular and cellular studies. *RND3* expression is down-regulated in breast invasive carcinoma, liver hepatocellular carcinoma and prostate adenocarcinoma. Indeed as we described earlier, depending on the tumour type, it may behave either as a tumour suppressor or as a tumour promoter [98]. These data were confirmed for hepatocellular carcinoma [109–111], and prostate adenocarcinoma [112,113], where *RND3* down-regulation is associated with poor

prognosis and favours malignant characteristics of tumour cells. Thus, *RND3* loss was associated with liver and prostate cancer cells proliferation, invasion, and chemoresistance [109,110,111,113,114]. Loss of Rnd3 was even found to favour epithelio-mesenchymal transition (EMT) by activating the Raf/MEK/ERK cascade and/or the ZEB2 EMT factor in hepatocellular carcinoma cells [107,109]. On the contrary, *RND3* up-regulation was described in gastric adenocarcinoma [23,115,116], non-small cell lung cancer [102,117,118,119] and melanoma [120,121,122], by both cellular and clinical approaches. In these malignancies, high *RND3* expression positively correlated with cancer cell aggressiveness and tumour progression. Concerning high-grade brain tumours, several studies reported *RND3* status in human glioblastoma multiform (GBM). *RND3* expression was described down-regulated by some [123], but found up-regulated by others [124,125], probably reflecting the heterogeneity and the complexity of these tumours. Early work performed in U87 cells reported that *RND3* up-regulation inhibited glioma cell proliferation and induced apoptosis [122]. More recently, Liu et al. also described *RND3* as a tumour suppressor in GBM. Its expression correlated positively with GBM patient survival, and is inversely associated with tumour size and tumour cell proliferation. They found that forced expression of Rnd3 inhibited GBM cell proliferation *in vitro* and tumour growth in mice by inhibiting Notch signalling activity [123]. In addition, the same group found downregulation of Rnd3 transcript and protein in human GBM samples, and this decrease correlated positively with E-Cadherin expression and negatively with the EMT marker, SNAIL1. They, thus, reported that *RND3* loss favours migration and invasion of GBM cells [126]. In addition, by acting on the NF- κ B signalling pathway, *RND3* was recently involved in the apoptosis of GBM cells [127]. In striking contrast, at least two other studies described *RND3* as a tumour promoter in glial cells [124,125]. In these studies, high Rnd3 levels correlated with poor prognosis and an increase in GBM cell proliferation and invasion.

Altogether, such data revealed that Rnd proteins are often altered in tumours and can act as both tumour suppressors and tumour promoting factors depending on the context. Whether the same tumour may jointly exhibit alteration of more than one Rnd family members remains to be studied. From the literature, it is clear that both *RND1* and *RND3* expressions are down-regulated in hepatocellular carcinoma supporting the rationale that Rnd1 and Rnd3 may act as tumour suppressor genes in liver cancer. Similarly, both *RND1* and *RND3* expressions are altered

in oesophageal squamous cell carcinoma. But, whereas Rnd1 is up-regulated in these tumours and promotes the growth and migration of cancer cells [104], Rnd3 is low and exerts tumour-suppressing effects [128,129]. Finally, in glioblastoma, besides the controversy for *RND3*, it looks like all three members are deregulated in those high-grade brain tumours (Figure 4b). Further studies are required to better understand the specific and redundant roles of Rnd proteins in malignancies.

5. Conclusions and future directions

Rnd proteins form a distinct group of the Rho family of small GTP-binding proteins with unusual biochemical properties. It consists of three proteins that play redundant functions in the regulation of the actin cytoskeleton and cell adhesion, but also exhibit unique and non-redundant roles in different tissues. Indeed, alterations in one of the three Rnds generate different pathologies. They were first described as endogenous antagonists of the RhoA/ROCK pathway; hence, their ability to inhibit the formation of contractile actomyosin cables. Thus, Rnd proteins regulate the actin cytoskeleton organization in several tissues. Moreover, Rnd proteins are now shown to act through different pathways increasing their mode of action. In the brain, they control multiple steps of neuronal development from proliferation to integration whereas in smooth muscles, they modulate contractility. To provide a better understanding of how these proteins regulate these functions, it will be important to identify novel partners for Rnd proteins, deciphering new pathways for Rnd1, Rnd2 and/or Rnd3. Indeed, proteins in general do not act as single units but perform cellular functions as components of complexes. The establishment of *Rnd3*-null mice allowed researchers to better understand the role of this member in cardiovascular diseases and neuronal functions. However, future studies should focus on the role of the two others Rnds in particular via the analysis of *Rnd1* or *Rnd2* knockout mice and conditional mutants. As a consequence of their important role in different cellular functions such as proliferation, migration and invasion, aberrant Rnd expression has been frequently observed in different types of cancer. Additional investigations on the molecular mechanisms are necessary to clarify the involvement of Rnd proteins in tumours. Interestingly, Rnd1 and Rnd3 are implicated in the chemotherapeutic resistance of cancer cells; so new research that could target Rnds is needed in order to expand our knowledge of anticancer agents.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

SB is supported by a post-doctoral fellowship from Fondation ARC. RA is supported by Rita Levi Montalcini funding from MIUR (Italian Ministry of Education, University and Research). EP's work was supported by ANR (ANR-19-CE16-0014-01). VM's work was supported by grants from La Ligue contre le Cancer comité de Gironde and from Fondation pour la Recherche Médicale (équipe FRM 2018); Agence Nationale de la Recherche [ANR-19-CE16-0014-01]; Association pour la Recherche sur le Cancer; Ligue Contre le Cancer [Comité Gironde]; Rita Levi Montalcini.

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